Supplemental Material

In Utero Exposure to Arsenic Alters Lung Development and Genes Related to Immune and Mucociliary Function in Mice

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Methods:

Thoracic gas volume and lung mechanics

To measure lung mechanics *in vivo*, mice were anaesthetised by intraperitoneal injection of a mixture containing xylazine (1mg/mL; Troy Laboratories, New South Wales, Australia) and ketamine (20 mg/mL; Troy Laboratories, New South Wales, Australia) at a dose 0.1 mL/10g body weight. Mice were tracheotomised with a 10 mm tracheal cannula (23G stainless steel) inserted and secured with suture. Mice were ventilated (MiniVent, Harvard Apparatus, Germany) at a tidal volume of 10 mL/kg, respiratory rate of 400 breaths per minute and positive end expiratory pressure of 2 cmH_2O.

For plethysmography, the trachea was occluded at end expiration (transrespiratory pressure, P_{rs} = 0 cmH_2O) and the intercostal muscles were stimulated with intramuscular electrodes to induce inspiratory efforts. Six 20V pulses of 2-3ms in duration were delivered over a 6s period while recording changes in tracheal pressure and plethysmograph box pressure. TGV was calculated using Boyle's law after correcting for the impedance and thermal properties of the plethysmograph (Janosi et al. 2006).

To measure lung mechanics using the forced-oscillation technique a forcing function (9 frequencies from 4 – 38 Hz) generated by a loudspeaker was delivered to the animal via a wave tube during pauses in ventilation (Sly et al. 2003). The respiratory system impedance spectrum (Z_{rs}) was measured and a 4-parameter model with constant phase tissue impedance was fitted to the data to partition Z_{rs} into components representing the

mechanical properties of the airways and parenchyma (Hantos et al. 1992). This model allowed the calculation of airway resistance (R_{aw}) and inertance (I_{aw}) and coefficients of tissue damping (G) and elastance (H). The resistance and inertance of the tracheal cannula were subtracted from R_{aw} and I_{aw} respectively. As most of the inertance is contained in the tracheal cannula, values of I_{aw} were insignificant and not reported.

Stereological analysis of lung structure

Lung structure was assessed used stereology techniques according to ATS/ERS guidelines (Hsia et al. 2010). Following euthanasia the tracheal cannula was instilled with 2.5% glutaraldehyde at 10 cmH_2O. This fixation pressure was chosen to fall within the range of volumes that lung function was measured i.e. at elastic-equilibrium lung volume (Zosky et al. 2010). Lungs were randomly oriented and embedded in paraffin wax (Nyengaard and Gundersen 2006). Starting at a random distance into the section (between 0 - 500μm), 5μm sections were taken at regular 500μm intervals throughout the lung and stained with haematoxylin and eosin. Lung volume was calculated using the Cavalieri method (Michel and Cruz-Orive 1988) and counting probes were used to obtain tissue/air volumes and alveolar surface area. Alveolar number was calculated using a physical dissector and Euler's number (Ochs 2006).

Quantification of mucous cells and protein in the airways

To detect CLCA3, MUC5B and REG3γ protein, an avidin-biotin-peroxidase complex method was used (Sabo-Attwood et al. 2005). Lung tissue sections were deparaffinised in xylene (3 x 5minutes) and rehydrated in isopropanol and graded ethanol (95 – 50%).

Slides were rinsed in deionised water and microwaved (900W, 2 minutes on high, 10 minutes on low) in 0.01M citric acid (pH 6.0) to allow antigen retrieval. Endogenous peroxidase activity was inhibited by incubating the slides in 1% H_20_2 followed by washes in phosphate-buffered saline (PBS) containing 1% H_20_2. Sections were blocked in PBS/Tween 20 containing 5% heat-inactivated normal goat serum. After washes, the sections were incubated with antibody α-p3b1 (Abcam, Cambridge, MA, USA) diluted 1:500 in PBS/Tween 20 containing 0.1% bovine serum albumin in a humidified chamber at 4ºC overnight. Sections were washed in PBS and incubated with biotinylated goat anti-rabbit immunoglobulins (5µg/ml, Vector Laboratories) diluted in PBS/Tween 20 containing 0.1% bovine serum in a humidified chamber at 4ºC overnight. Colour was developed for 60 minutes using freshly prepared avidin-biotin-peroxidase complex solution (Vectastain Elite ABC kit, Vector Laboratories) diluted in PBS/Tween 20 with 0.1% bovine serum albumin, followed by repeated washes in PBS, and rinsing in water. The slides were counterstained with hematoxylin, dehydrated through ascending graded ethanol, cleared in xylene, and coverslipped before examination by light microscopy.

References

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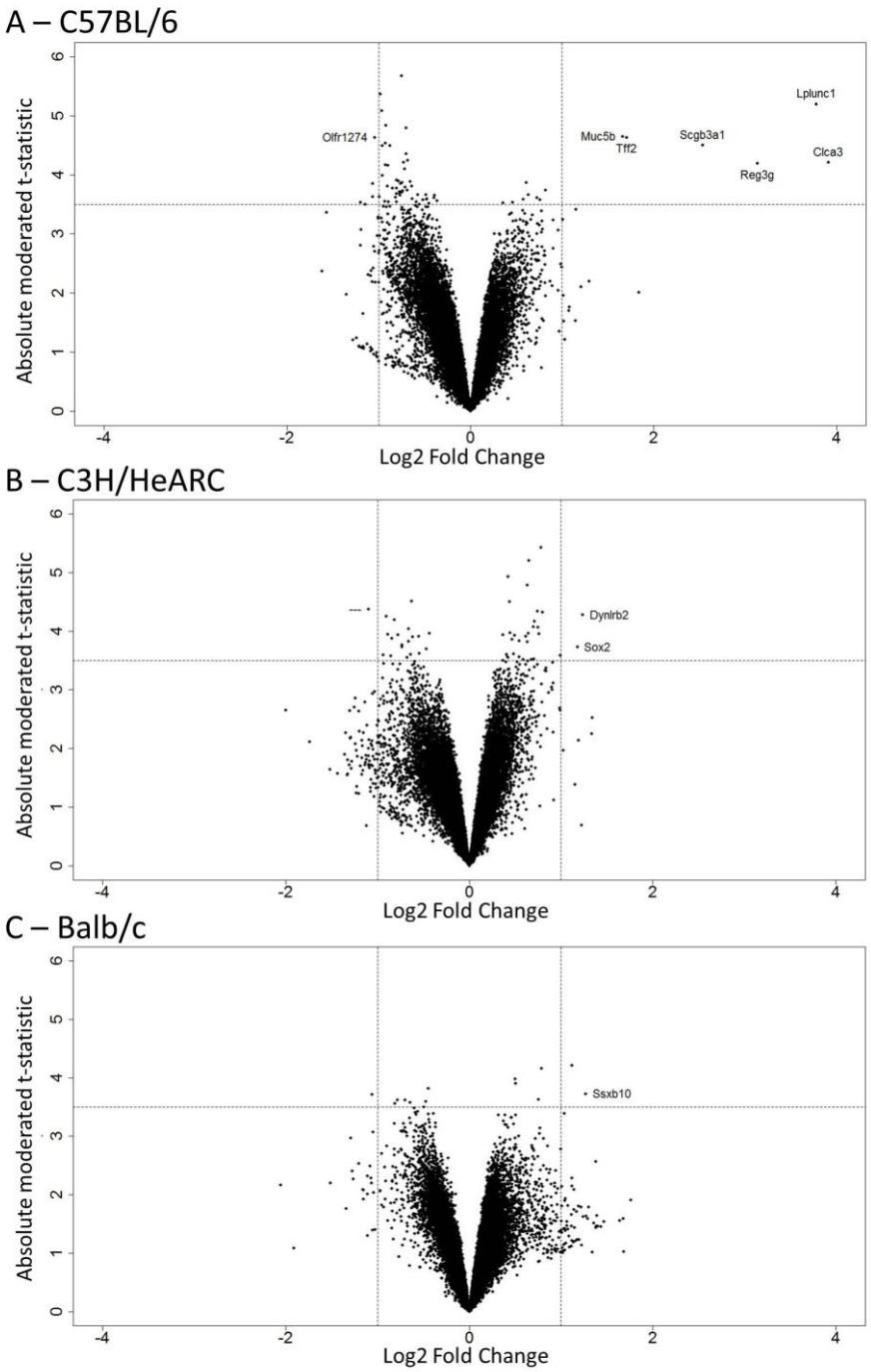
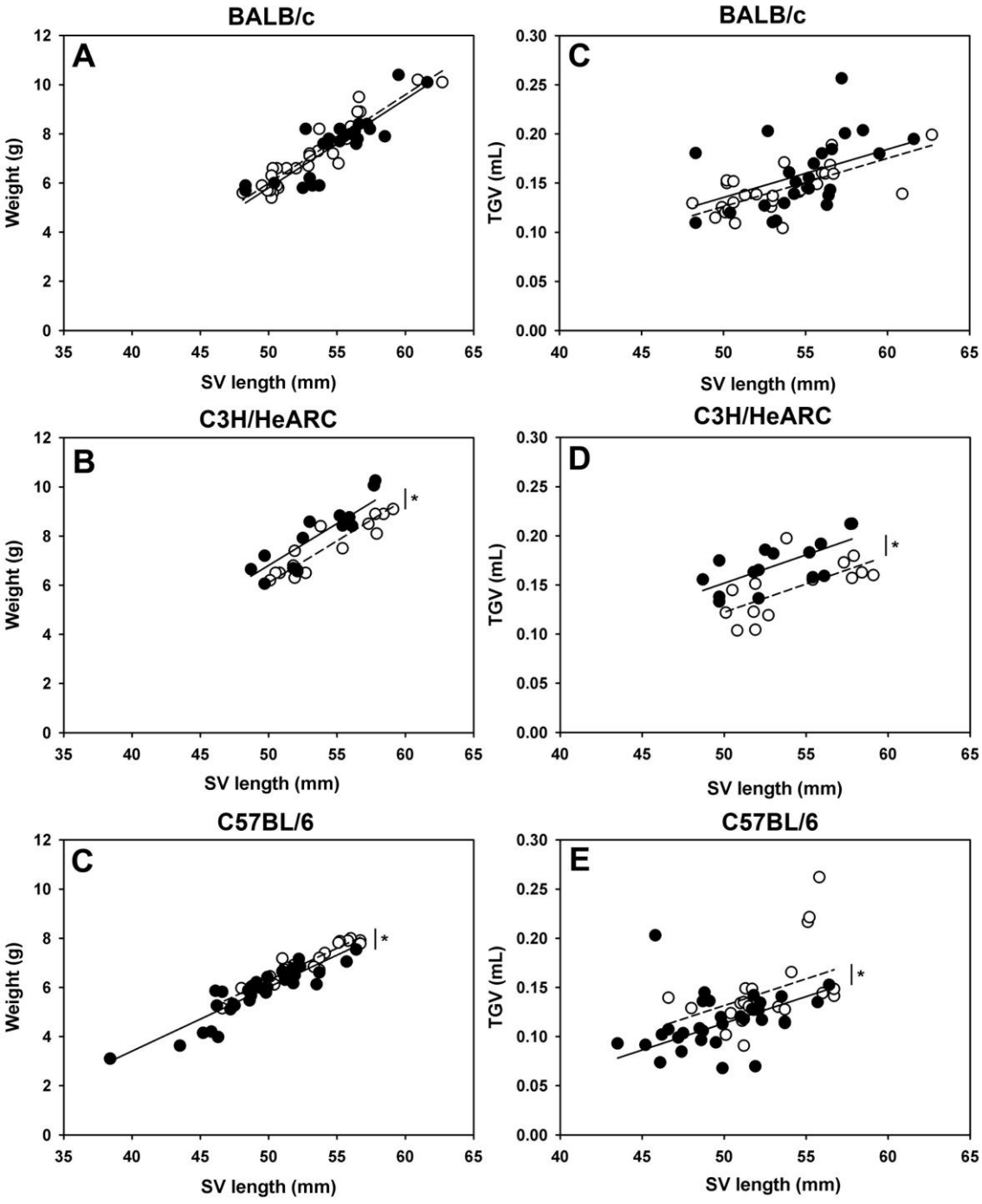
Supplemental Material, Table S1: Maternal and birth outcomes. Outcomes for BALB/c, C3H/HeARC and C57BL/6 dams and their offspring exposed to 100µg/L arsenic via drinking water or control water from d8 gestation to birth (mean ± SD). There were no differences in maternal water consumption, gestation period or litter size between control and arsenic exposed mice. C57BL/6 offspring exposed to 100µg/L arsenic were born significantly smaller than control mice in both weight and length. (* indicates significantly different to control, $p < 0.05$).

	BALB/c		**C3H/HeARC**		**C57BL/6**	
	Control	Arsenic	Control	Arsenic	Control	Arsenic
Maternal water consumption (mL/day)	4.43 ± 0.62	5.32 ± 1.34	6.88 ± 1.75	6.09 ± 0.65	5.83 ± 1.38	5.89 ± 1.39
Gestation period (days)	19.7 ± 0.52	19.6 ± 0.55	19.8 ± 0.50	19.3 ± 0.50	19.8 ± 0.45	19.5 ± 0.93
Litter size (pups/dam)	5.67 ± 3.39	5.00 ± 1.87	4.25 ± 2.22	4.50 ± 1.73	6.00 ± 0.82	6.62 ± 3.02
Birth weight (g)	1.32 ± 0.22	1.40 ± 0.19	1.46 ± 0.24	1.55 ± 0.11	1.40 ± 0.18	1.18 ± 0.17*****
Birth length (mm)	28.4 ± 1.63	28.9 ± 1.36	29.4 ± 2.02	29.5 ± 1.25	28.7 ± 1.62	27.2 ± 1.60*****



Supplemental Material, Figure S1: Microarray volcano plots. Volcano plots show clusters of differentially expressed genes in response to arsenic in C57BL/6 (A), C3H/HeARC (B) and BALB/c (C) mice. Annotated genes with an absolute t-statistic greater than 3.5 and fold change greater than 2 were considered differentially expressed.



Supplemental Material, Figure S2: Somatic growth and thoracic gas volume outcomes. Body weight and thoracic gas volume (TGV) plotted against body (SV) length in two week old offspring from BALB/c, C3H/HeARC and C57BL/6 mice exposed to 100 µg/L arsenic (closed circles) via drinking water or control water (open circles) from d8 gestation to birth. (* indicates significantly different to control, $p < 0.05$).